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Development of PCR Assays Targeting the Genes Involved in Synthesis and Assembly of the New *Escherichia coli* O174 and O177 O Antigens

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***Escherichia coli* O174 and O177 are newly described O serogroups which were reported as human pathogens. Identification of these strains by serotyping has been restricted, as the required sera are not commercially available. In this study, a collection of 13 *E. coli* O174 strains and 12 *E. coli* O177 strains was studied on the O:H serotypes and virulence markers. The O-antigen gene clusters of *E. coli* O174 and O177 were sequenced, and associated genes were assigned functions on the basis of homology. Two genes, each specific for *E. coli* O174 and O177, were identified. PCR assays based on the O-antigen-specific genes were developed and tested on 25 clinical and environmental isolates of those two serogroups as well as 26 isolates of other O serogroups. As little as 1 pg per μl of chromosomal DNA and as few as 0.1 CFU per g of pork and water samples were detected for either strain. The PCR assays established in this study were shown to be highly sensitive and reliable and could be the method of choice for detection of these two human pathogens from clinical, food, and other environmental samples.**

Escherichia coli is a species that includes both commensal and pathogenic clones of strains, which are normally identified by serological typing of their O (lipopolysaccharide), H (flagellar), and, in some cases, K (capsular) surface antigens. At present, 176 O and 53 H antigens, which can occur in different combinations in wild-type isolates of strains, have been described for *E. coli* (17, 20, 23). *E. coli* clones causing disease in humans, such as enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC), are frequently associated with certain O:H types, for example, EHEC O157:H7 and EPEC O55:H6 strains. A growing number of *E. coli* serotypes are associated with the EPEC or EHEC group, and among them are the newly described O serogroups O174 (formerly designated as OX3 or OX174) and O177 (formerly called OX177) (3, 10, 23, 24). Typing sera for detection of the eight novel *E. coli* O groups from O174 to O181 are not commercially available, and their serological detection is restricted to a small number of *E. coli* reference laboratories. Studies from reference centers indicate that domestic animals serve as a reservoir for these strains, which were reported as human pathogens in different countries and on different continents (3, 8, 19, 21, 23, 25, 26).

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More recently, DNA-based typing methods were employed for the detection of *E. coli* O-antigen-encoding genes, and correspondent PCR protocols for the identification of pathogenic *E. coli* strains such as O157, O111, O114, and O172 were developed (12, 14, 28, 30). The O antigen, as a part of the lipopolysaccharide moiety of gram-negative bacteria, consists of many repeats of an oligosaccharide unit (O unit) (9). The genes for O-antigen synthesis are normally clustered between the two housekeeping genes *galF* and *gnd* on the *E. coli* chromosome, and a conserved 39-bp JUMPStart sequence, which is required for the regulation of downstream genes by RfaH (15), is located in the intergenic region between *galF* and the O-antigen gene cluster (18). The genes in the O-antigen gene cluster are normally classified into three main classes: genes for synthesis of nucleotide sugar precursors, genes encoding glycosyltransferases, and genes for O-unit processing, including the flippase (Wzx) and polymerase (Wzy) genes (9). Genes in the last two classes are O-antigen specific since they are specific to sugar donors, sugar acceptors, and linkages between the sugars (30). The differences between O-antigen forms are almost entirely due to genetic variations in the O-antigen gene clusters (9).

PCR methods for the detection of O-antigen-specific genes such as *wzx* and *wzy* represent a reliable, rapid, and sensitive alternative to serotyping, particularly for *E. coli* strains that belong to serogroups that are not covered by the panel of commercially available antisera (5, 14, 22, 27). Moreover, PCR detection of O-antigen-specific genes allows detection of

TABLE 1. Relevant properties of *E. coli* O174 and O177 strains

Strain no.	Strain (reference)	Serotype[a]	Origin (yr of isolation)	VCA[b]	Hly[c]	Positive for virulence gene[d]	Disease[e]
G1609	2531-54 (23)	O174:H27	Human; *E. coli* O174 reference strain	−	None	None	D
G1610	CB9135 (25)	O174:[H8]	Sheep, Norway (2001)	+	None	stx_{1c}, stx_{2c}	AS
G1611	CB9534	O174:[H8]	Raw Cheese, France (2002)	+	Ehly	*ehxA*, stx_{1c}	None
G2032	CB8293 (3)	O174:H16	Human, Germany (1999)	+	None	stx_{2c}	ND
G2033	CB9043 (25)	O174:H8	Sheep, Norway (2001)	+	Ehly	*ehxA*, stx_{1c}, stx_{2c}	AS
G2034	CB8377	O174:H2	Ground meat, Germany (2000)	+	Ehly	*ehxA*, stx_1, stx_2	None
G2035	CB7950 (3)	O174:H2	Human, Germany (1999)	+	Ehly	*ehxA*, stx_1, stx_2	BD
G2036	CB8958	O174:H21	Human, Germany (2001)	+	None	stx_{2c}	D
G2037	CB7855 (3)	O174:H21	Human, Germany (1998)	+	Ehly	*ehxA*, stx_{2d}	BD
G2038	CB7033 (3)	O174:H2	Human, Germany (1997)	+	Ehly	*ehxA*, stx_1, stx_2	BD
G2039	CB9042 (25)	O174:H8	Sheep, Norway (2001)	+	Ehly	*ehxA*, stx_{1c}, stx_{2c}	AS
G2040	CB8185	O174:H28	Minced meat, Germany (1999)	+	Ehly	*ehx*A, stx_2	None
G2041	CB7398	O174:H43	Human, France (1995)	−	None	None	HUS[f]
G1606	E40874-85 (23)	O177:H25	Cattle; *E. coli* O177 reference strain	+	None	stx_2, *eae*-β	ND
G1607	CB9861	O177:H11	Cattle, Brazil (2003)	−	Ehly	*eae*-β1, *ehxA*	AS
G1608	CB9268	O177:H11	Cattle, Germany (1990)	+	Ehly	*ehxA*, stx_2, *eae*-β	AS
G2042	CB7137	O177:H25	Cattle, Switzerland (1997)	+	Ehly	*ehxA*, stx_2, stx_{2c}, *eae*-β	AS
G2043	CB7135	O177:[H25]	Cattle, Switzerland (1997)	+	Ehly	*ehxA*, stx_{2c}, *eae*-β	AS
G2044	CB9714	O177:H25	Human, Germany (2003)	−	Ehly	*eae*-β, *ehxA*	D
G2045	CB1138	O177:H11	Calf, Germany (1990)	−	Ehly	*eae*-β, *ehxA*	D
G2046	CB1139	O177:H11	Calf, Germany (1990)	−	Ehly	*ehxA*, *eae*-β	D
G2047	CB8989	O177:[nt]	Human, Germany (2001)	+	None	stx_1, *eae*-α	D
G2048	CB8995	O177:[nt]	Human, Germany (2001)	+	None	stx_1, *eae*-α	D
G2049	CB7134	O177:[H25]	Cattle, Switzerland (1997)	+	Ehly	*ehxA*, stx_2, stx_{2c}, *eae*-β	AS
G2068	CB9657	O177:H26	Human, Thailand (2003)	−	None	*eae*-β	D

[a] In *fliC* genotypes of nonmotile strains, [nt] indicates that a serotype could not be assigned to known *fliC* RFLP type.
[b] VCA, Vero cell toxicity assay.
[c] Hemolytic phenotype. Ehly, enterohemolytic (6).
[d] Virulence markers investigated by PCR. *ehxA*, EHEC hemolysin A-encoding gene; *stx*, Shiga toxin-encoding gene; *eae*, intimin-encoding gene; EAF plasmid, plasmid-specific 397-bp region.
[e] AS, no signs of disease; D, nonbloody diarrhea; BD, bloody diarrhea; HUS, hemolytic uremic syndrome; ND, no data.
[f] Strain was originally reported to carry an stx_2 gene (F. Grimont, Institut Pasteur, Paris, France, personal communication).

strains which are serologically rough for their O antigens, and last but not least, serological cross-reactions between different O antigens that may cause difficulties in O typing are ruled out.

In this study, a collection of 13 *E. coli* O174 strains and 12 *E. coli* O177 strains was studied on the O:H serotypes and virulence markers. The O-antigen gene clusters of the *E. coli* reference strains for the newly defined serogroups O174 and O177 were sequenced, and the associated genes were identified on the basis of homology. By screening against all 186 *E. coli* O serotypes (including *Shigella*) strains, genes specific for *E. coli* serogroups O174 and O177 were identified. The PCR assays that were developed in this study for detection of O-antigen-specific genes were shown to be highly specific and sensitive and were shown to be useful for the detection of *E. coli* O174 and O177 strains from clinical, food, and environmental samples.

MATERIALS AND METHODS

Bacterial strains. The reference strains for *E. coli* O174 (2531-54) and O177 (E40874-85) used for nucleotide sequence analysis of their O-antigen clusters were described elsewhere previously (23). A collection of 13 *E. coli* O174 and 13 O177 strains which were characterized for their serotypes, phenotypical traits, and virulence markers was used for evaluation of the O-antigen gene-specific PCR (see below). The strains originated from feces of humans and animals and from food and were collected at different time periods and geographical locations investigated (Table 1). Other *E. coli* and *Shigella* type strains used in this study were previously described (12).

Serotyping and molecular typing of flagellar (*fliC*) genes. Serotyping of *E. coli* O and H antigens was performed as described previously (5). Specific antisera for detection of O antigens O174 and O177 were produced at the Robert Koch Institute with reference strains 2531-54 (O174:H27) and E40874-85 (O177:H25), respectively (Table 1). Agglutination reactions were performed in twofold dilution steps of antiserum with boiled bacteria as described previously (20) Agglutinating titers with homologous strains were 1:3,200, and no significant cross-reactions (<1:200) were observed with other *E. coli* O antigens. The O174 and O177 test strains did not differ for more than 1 titer step in their agglutination reactions compared to their respective reference strains. Nonmotile *E. coli* strains were investigated for their flagellar (*fliC*) genes by PCR and analysis of HhaI restriction fragment length polymorphisms (RFLPs) of PCR products as described previously (4).

Phenotypical and genotypical characterization of virulence markers. The *E. coli* O174 and O177 strains were investigated for the production of cytotoxins with the Verocell assay and for hemolysins on enterohemolysin agar (Oxoid, Wesel, Germany) (6). Detection and subtyping of genes encoding the EPEC adherence factor (EAF) plasmid, intimins (*eae*), Shiga toxins (*stx*), and EHEC-hemolysin (*ehxA*) were performed by PCR and RFLP analysis using primers and restriction enzymes described previously (3, 13). An updated nomenclature of Shiga toxin types was used as described recently (3).

Construction of DNase I shotgun bank, sequencing, and analysis of O-antigen gene cluster. Chromosomal DNA was prepared as previously described (2). The primer pairs wl-1098 (5′-ATT GGT AGC TGT AAG CCA AGG GCG GTA GCG T-3′) and wl-2211 (5′-CAC TGC CAT ACC GAC GAC GCC GAT CTG TTG CTT GG-3′), based on the JUMPStart sequence and *gnd*, respectively (30), were used to amplify the O-antigen gene clusters of *E. coli* O174 and O177 type strains 2531-54 and E40874-85 (Table 1). The PCR was performed for 30 cycles as follows: a denaturation step at 94°C for 10 s, an annealing step at 60°C for 30 s, and an extension step at 68°C for 15 min. Shotgun banks for each strain were constructed as described previously (30). Sequencing was carried out using an ABI 3730 automated DNA sequencer, and sequence data were analyzed using computer programs as described previously (11).

Specificity and sensitivity test of O-serogroup-specific PCR assay. Chromosomal DNA was prepared from each of 186 type strains to represent a broad range of O antigens of *E. coli* and *Shigella* and was used to make DNA pools as described previously (12). A total of 13 pools were made, each containing DNA

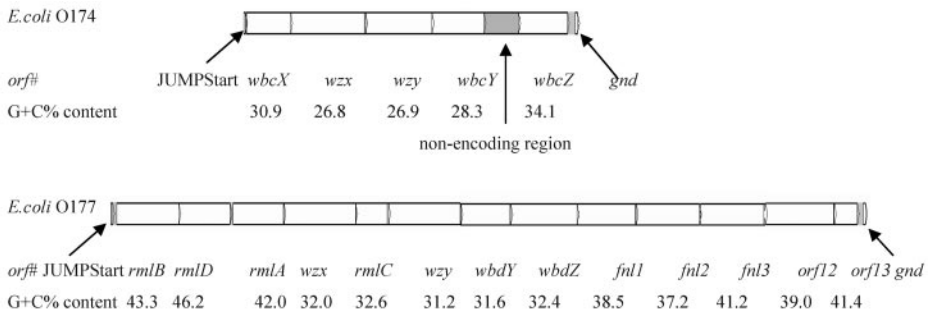

FIG. 1. O-antigen gene clusters of *E. coli* O174 and *E. coli* O177. All genes are transcribed in a direction from the JUMPStart sequence to *gnd*.

from 12 to 19 strains (12). Either *E. coli* strain 2531-54 (O174) or strain E40874-85 (O177) and the strains of pool 12 were used to make up pool 13. Primer pairs (see Table 4) specific for *wzx* and *wzy* genes of *E. coli* O174 and O177 were used to screen the DNA pools. The PCR was performed as follows: a denaturation step at 95°C for 30 s, an annealing step at 45°C for 30 s, and an extension step at 72°C for 1 min for 30 cycles. Template DNA from 50 clinical *E. coli* isolates including 12 *E. coli* O174, 11 *E. coli* O177 (Table 1), and 26 *E. coli* strains belonging to other O serogroups (data not shown) were prepared as described previously (14) and screened with the primers listed in Table 4 in a double-blind test.

The sensitivity of the PCR assays was tested with 10-fold serial dilutions of chromosomal DNA of *E. coli* strain 2531-54 (O174) or E40874-85 (O177) with the primer pairs designed (see Table 4). Primer pairs wl-4397/wl-4398 and wl-4399/wl-4400 from *E. coli* O174 and wl-4393/wl-4394 and wl-4395/wl-4396 from *E. coli* O177 were also used to screen for *E. coli* strains of these two O serogroups in pork and water samples using a method described previously (14).

Nucleotide sequence accession numbers. The DNA sequences of the *E. coli* O174 and O177 O-antigen gene clusters have been deposited in GenBank under the accession numbers DQ008592 and DQ008593, respectively.

RESULTS AND DISCUSSION

Serotypes and virulence markers of *E. coli* O174 and O177 strains. The 13 *E. coli* O174 strains originated from diseased humans, healthy sheep, and food. They were distributed over seven H types (H2, H8, H16, H21, H27, H28, and H43) (Table 1). All strains were negative for an intimin-encoding gene (*eae*), which is in accordance with previous investigations made with the *E. coli* O174 group (3, 7, 8, 19, 25). Twelve of the O174 strains from our study produced Shiga toxins, and toxin types 1 and 2 were associated with O174:H2 and O174:H28 strains, types 1c and 2c were associated with O174:H8 and O174:H16 strains, and type 2d was associated with O174:H21 strains. Eight of the O174 strains showed an enterohemolytic phenotype and carried an *ehxA* gene, which is a typical trait of many EHEC strains (3). Toxin type 2 *E. coli* O174:H2 and type 2d O174:H21 strains were associated with bloody diarrhea in humans. Studies of *E. coli* O174 in animals have indicated that cattle are a reservoir for O174:H2 and O174:H21 strains and that sheep are a reservoir for O174:H8 strains (7, 21, 25).

The 11 *E. coli* O177 strains originated from diarrheagenic humans and calves as well as from healthy cattle. They were distributed over three H types (H11, H25, and H26), except for two nonmotile strains, which could not be typed for their *fliC* genes (Table 1). In contrast to *E. coli* O174, all *E. coli* O177 strains investigated were positive for an *eae* gene. Those distributed over H types H11, H25, and H26 carried an *eae*-β gene, and the two nonmotile strains carried an *eae*-α gene. Seven of the *E. coli* O177 strains produced Shiga toxins (types 1 and/or 2). Nine were positive for enterohemolysin and the *ehxA* gene. According to their virulence profiles, *E. coli* O177:H11 and O177:H25 were associated with two pathotypes such as atypical EPEC (*eae* positive) and EHEC (*eae* and *stx* positive) (24). None of the *E. coli* O177 and O174 strains were positive for sequences of the EAF plasmid, which is harbored by typical (class I) EPEC strains (24).

Nucleotide sequence analysis of the *E. coli* O174 O-antigen gene cluster. A sequence of 5,660 bases between the JUMPStart sequence and *gnd* was obtained from *E. coli* O174, and five open reading frames (ORFs) were found (Fig. 1). By comparing these to related genes in nucleotide sequence databases, all ORFs were assigned functions and shown to be related to O-antigen biosynthesis (Table 2).

(i) O-unit-processing genes. Both Wzx and Wzy are typical inner membrane proteins with more than nine transmembrane segments, and Wzy also contains a large periplasmic loop of more than 30 amino acids. Orf2 has 11 predicted transmembrane segments and shares 49% similarity to Wzx of *Yersinia*

TABLE 2. Putative genes in the *E. coli* O174 O-antigen gene cluster

Gene name	Location in sequence	G+C content (%)	Similar protein(s), species (GenBank accession no.)	% Identical aa/% similar aa (no. of aa overlap)[a]	Putative function of protein
wbcX	51–794	30.9	LgtD, *Rickettsia prowazekii* (CAA14903)	6/43 (209)	Glycosyltransferase
wzx	797–2068	26.8	Wzx, *Yersinia enterocolitica* (type 0:8) (AAC60766)	27/49 (381)	O-unit flippase
wzy	2049–3188	26.9	Wzy, *Bacillus cereus* ATCC 10987 (AAS44289)	24/46 (243)	O-unit polymerase
wbcY	3191–4075	28.3	Putative glycosyltransferase, *Bacillus halodurans* C-125 (BAB07432)	32/55 (232)	Glycosyltransferase
wbcZ	4661–5482	34.1	Putative glycosyltransferase, *Salmonella enterica* (AAG09520)	52/70 (265)	Glycosyltransferase

[a] aa, amino acid.

TABLE 3. Putative genes in the *E. coli* O177 O-antigen gene cluster

Gene name	Location in sequence	G+C content (%)	Similar protein(s), strain(s) (GenBank accession no.)	% Identical aa/% similar aa (no. of aa overlap)[a]	Putative function of protein
rmlB	108–1193	43.3	RmlB, *Escherichia coli* K-12 (AAC75102)	97/98 (361)	dTDP-D-glucose-4,6-dehydratase
rmlD	1193–2092	46.2	RmlD, *Escherichia coli* K-12 (AAC75101)	97/98 (299)	dTDP-6-deoxy-L-mannose-dehydrogenase
rmlA	2150–3040	42.0	RmlA, *Escherichia coli* K-12 (AAC75100)	91/96 (290)	Glucose-1-phosphate thymidylyltransferase
wzx	3030–4295	32.0	Wzx, *Shigella flexneri* 2a strain 301 (AAN43639)	51/74 (407)	O-unit flippase
rmlC	4308–4874	32.6	RmlC, *Escherichia coli* K-12 (AAN60457)	65/79 (174)	dTDP-6-deoxy-D-glucose-3,5-epimerase
wzy	4855–6141	31.2	Wzy, *Shigella flexneri* 2a strain 301 (AAN43636)	25/52 (175)	O-unit polymerase
wbdY	6138–7010	31.6	Rhamnosyltransferase I, *Shigella dysenteriae* type 1 (AAA16937)	30/48 (294)	Glycosyltransferase
wbdZ	6982–8169	32.4	Glycosyltransferase, *Shigella boydii* type 13 (AAR24277)	26/45 (398)	Glycosyltransferase
fnlA	8174–9208	38.5	Fnl1, *Escherichia coli* O26 (AAN60461)	92/98 (344)	L-Fucosamine synthetase
fnlB	9192–10316	37.2	Fnl2, *Escherichia coli* O26 (AAN60462)	96/98 (374)	L-Fucosamine synthetase
fnlC	10331–11443	41.2	Fnl3, *Escherichia coli* O26 (AAN60463)	98/98 (370)	L-Fucosamine synthetase
wbuB	11443–12651	39.0	Putative L-fucosamine transferase, *Escherichia coli* O26 (AAN60464)	96/97 (402)	L-Fucosamine transferase
wbuC	12642–13049	41.4	WbuC, *Escherichia coli* O26 (AAN60465)	98/100 (135)	Unknown

[a] aa, amino acid.

enterocolitica (type 0:8). It belongs to the protein family PF01943 (E value, $1.4 \times e^{-5}$), and members of this family are involved in the export of the O antigen and teichoic acid. Therefore, orf2 was proposed to be an O-unit flippase gene (*wzx*) and was named accordingly. Orf3 has 12 predicted transmembrane segments and a large periplasmic loop of 61 amino acid residues. It shares 46% similarity to the putative Wzy of *Bacillus cereus*. Therefore, orf3 was proposed to be an O-antigen polymerase gene (*wzy*) and was named accordingly.

(ii) Glycosyltransferase genes. Orf1, Orf4, and Orf5 belong to the glycosyltransferase family 2 (pfam00535 E values, $5 \times e^{-5}$, $1.8 \times e^{-13}$, and $6.0 \times e^{-25}$, respectively) and share 43 to 70% similarity with other putative glycosyltransferases (Table 2). Therefore, orf1, orf4, and orf5 were proposed to be glycosyltransferase genes and were named *wbcX*, *wbcY*, and *wbcZ*, respectively.

The O-antigen structure of *E. coli* O174 has not been characterized. The absence of sugar synthesis genes in the O-antigen gene cluster indicates that the O unit of *E. coli* O174 consists of common sugars such as Glc, Gal, GlcNAc, and GalA. Genes for the synthesis of common sugars are outside the O-antigen gene cluster (9). Glycosyltransferase genes are highly specific to sugar donors, sugar receptors, and the linkages between them. The *wecA* gene, which is located outside the O-antigen gene cluster, is responsible for the transfer of GlcNAc presented as the first sugar in most *E. coli* O units (1). Three glycosyltransferase genes were found in the *E. coli* O174 O-antigen gene cluster. Therefore, *E. coli* O174 O antigen was predicted to be composed of tetrasaccharide repeating units.

A large nonencoding region between orf4 and orf5 (positions 4084 to 4668) was found. It contains numbers of stop codons and shares no similarity to other genes in the databases. It appears that this intergenic region underwent numerous changes under selection pressure.

Nucleotide sequence analysis of the *E. coli* O177 O-antigen gene cluster. A sequence of 13,198 bases between the JUMPStart sequence and *gnd* was obtained from *E. coli* O177, and 13 ORFs were found (Fig. 1). By comparing these to related genes in nucleotide sequence databases, all ORFs were assigned functions and were shown to be related to O-antigen biosynthesis (Table 3).

(i) Sugar synthesis genes. Orf1, Orf2, Orf3, and Orf5 share 97, 97, 91, and 65% identity to RmlB, RmlD, RmlA, and RmlC, respectively, of the *E. coli* K-12 O-antigen gene cluster. They also share high-level identity to other known Rml proteins, RmlB, RmlD, RmlA, and RmlC, of *E. coli* and *Shigella* strains (data not shown). The Rml proteins are enzymes for the synthesis of dTDP-L-rhamnose and have been well characterized in many bacterial strains. orf1, orf2, orf3, and orf5 were identified as *rmlB*, *rmlD*, *rmlA*, and *rmlC*, respectively. The presence of the *rml* genes indicates that the *E. coli* O177 O antigen contains a rhamnose moiety. In most *E. coli* and *Shigella* strains, the *rmlABCD* genes are arranged as a group at the 5′ end of the O-antigen gene cluster. However, in *E. coli* O177, the *rmlC* gene is separated from other *rml* genes by *wzx*. The same gene order was found in the *Shigella boydii* type 9 O-antigen gene cluster (29). As in the case of *S. boydii* type 9, the atypical position of *rmlC* also indicates that the *E. coli* O177 O-antigen gene cluster was assembled more recently by lateral transfer.

Orf9, Orf10, and Orf11 share 80, 55, and 70% identity to FnlA (WbjB), FnlB (WbjC), and FnlC (WbjD) of the *Pseudomonas aeruginosa* O11 O-antigen gene cluster, respectively, which were experimentally identified as UDP-L-FucNAc biosynthesis pathway enzymes (16). They also share 92, 96, and 98% identity to FnlA (Fnl1), FnlB (Fnl2), and FnlC (Fnl3) of the *E. coli* O26 O-antigen gene cluster, respectively. Therefore, orf9, orf10, and orf11 were identified as *fnlA*, *fnlB*, and *fnlC* and were named accordingly. The presence of the *fnl* genes indicates that the *E. coli* O177 O antigen contains a fucosamine moiety.

(ii) O-unit-processing genes. Orf4 has 10 predicted transmembrane segments and shares 74% similarity to Wzx of *S. flexneri* 2a strain 301. It belongs to the Pfam family PF01943 (E

TABLE 4. Primers used for PCR detection of strains of *E. coli* O serogroups O174 and O177

Serogroup	Gene	Base positions	Forward primer/reverse primer	Length of PCR product (bp)
O174	*wzx*	805–2076	wl-4397 (positions 891–908) (5′-TCTAGGACCTGTAGAACA-3′)/wl-4398 (positions 1529–1546) (3′-GTAGTTGATCTGAGCGAT-5′)	656
			wl-4421 (positions 1479–1498) (5′-AGTAATGATCGGAACTATGC-3′)/wl-4422 (positions 1861–1878) (3′-TTTTGGAATAACCGTCGT-5′)	400
	wzy	2057–3196	wl-4399 (positions 2421–2439) (5′-TATGGGTCCTATTACTTTC-3′)/wl-4400 (positions 3160–3179) (3′-GTATCGGAGATCATTATTAC-5′)	759
			wl-4423 (positions 2512–2529) (5′-TCTAGGGACTGTTGTTAC-3′)/wl-4424 (positions 2944–2961) (3′-TCTTTAACCCGACTTATC-5′)	450
O177	*wzx*	3030–4295	wl-4393 (positions 3528–3545) (5′-GTTGCGTTGCCTGCTGTA-3′)/wl-4394 (positions 4190–4207) (3′-GGTAAAGCCCTATCATCC-5′)	680
			wl-4417 (positions 3170–3187) (5′-TATGGCATTAGTGGGTTA-3′)/wl-4418 (positions 3532–3549) (3′-GCAACGGACGACATTCAT-5′)	380
	wzy	4855–6141	wl-4395 (positions 4946–4963) (5′-TTTTATTAGGGTCAGGAG-3′)/wl-4396 (positions 5419–5436) (3′-CACAACGACGGATTATCA-5′)	491
			wl-4419 (positions 5386–5403) (5′-ATTATTGCCGATACACCG-3′)/wl-4420 (positions 5798–5815) (3′-CAGGACAAGACCCATGAC-5′)	430

value, $3.1 \times e^{-52}$), and members of this family are involved in the export of the O antigen and teichoic acid. Therefore, orf4 was proposed to be an O-unit flippase gene (*wzx*) and was named accordingly. Orf6 has 10 predicted transmembrane segments and a large periplasmic loop of 56 amino acid residues. It also shares 52% similarity to Wzy of *S. flexneri* 2a. Therefore, orf6 was proposed to be an O-antigen polymerase gene (*wzy*) and was named accordingly.

(iii) Glycosyltransferase genes. Orf7 and Orf8 belong to glycosyltransferase family 2 (PF00535; E value, $1.7 \times e^{-2}$) and glycosyltransferase family 1 (PF00534; E value, $6.6 \times e^{-7}$). They also share 48 and 45% similarity to glycosyltransferases of *Shigella dysenteriae* type 1 and *Shigella boydii* type 13, respectively. orf7 and orf8 were proposed to be glycosyltransferase genes and were named *wbdY* and *wbdZ*, respectively. Orf12 belongs to glycosyltransferase family 1 (PF00534; E value, $4.5 \times e^{-3}$) and shares 96% identity to a putative L-fucosamine transferase, WbuB, of *E. coli* O26 (9); based on this high-level identity, orf12 was proposed to be an L-Fucosamine transferase gene and was named *wbuB*.

(iv) Nonfunctional gene. Orf13 shares 98% identity to a proposed remnant gene product, WbuC, of *E. coli* O26 (9). *orf13* was proposed to be nonfunctional and was named *wbuC*.

The O-antigen structure of *E. coli* O177 has not been characterized. Based on the number of glycosyltransferase genes found in the O-antigen gene cluster, *E. coli* O177 O antigen was predicted to be composed of tetrasaccharide O-unit containing rhamnose and FucNAc.

Specific identification of *E. coli* O174 and O177 serogroup-specific genes by PCR. Two pairs of primers specific for *wzx* and *wzy* genes were designed for *E. coli* O174 (Table 4) and used to screen DNA pools containing representatives of the

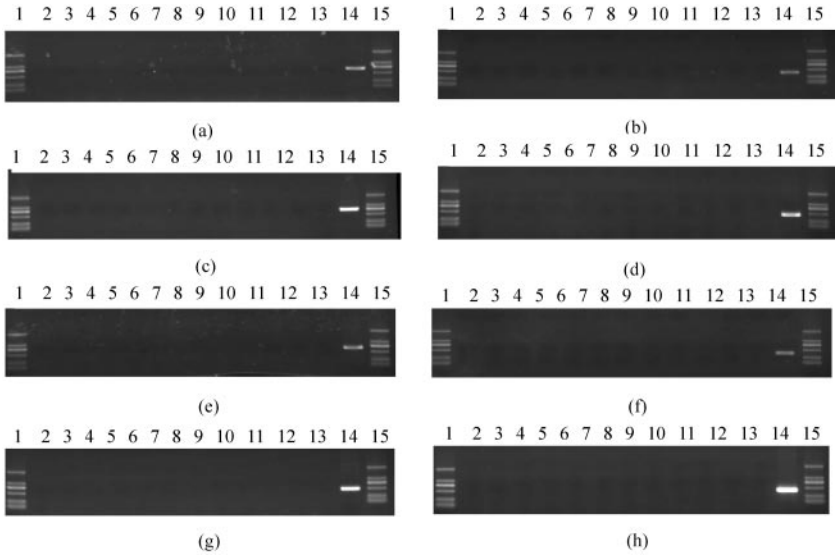

FIG. 2. Amplification products obtained by PCR of 13 pools of DNA including 186 *E. coli* and *Shigella* O-antigen type strains and *E. coli* O174 strain G1609 or *E. coli* O174 strain G1606 using primer pairs wl-4397/wl-4398 (a), wl-4421/wl-4422 (b), wl-4399/wl-4400 (c), wl-4423/wl-4424 (d), wl-4393/wl-4394 (e), wl-4417/wl-4418 (f), wl-4395/wl-4396 (g), and wl-4419/wl-4420 (h). Lanes 1 and 15, DNA marker, bands with lengths of 100 bp, 250 bp, 500 bp, 750 bp, 1 kb, and 2 kb; lanes 2 to 14, pool 1 to pool 13, respectively.

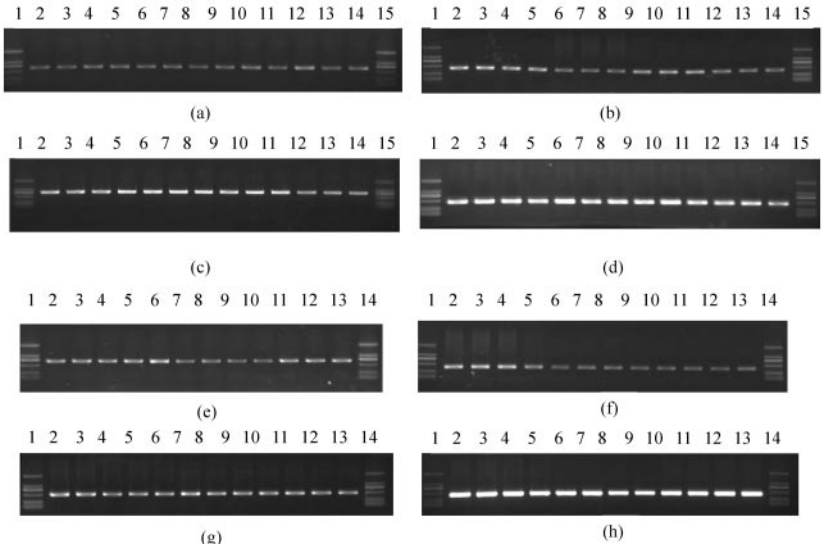

FIG. 3. Amplification products obtained by PCR of 13 clinical isolated *E. coli* O174 strains using primer pairs wl-4397/wl-4398 (a), wl-4421/wl-4422 (b), wl-4399/wl-4400 (c), and wl-4423/wl-4424 (d). Lanes 1 and 15, DNA marker, bands with lengths of 100 bp, 250 bp, 500 bp, 750 bp, 1 kb, and 2 kb; lane 2, G1609; lane 3, G1610; lane 4, G1611; lane 5, G2032; lane 6, G2033; lane 7, G2034; lane 8, G2035; lane 9, G2036; lane 10, G2037; lane 11, G2038; lane 12, G2039; lane 13, G2040; lane 14, G2041. Also shown are amplification products obtained by PCR of 12 clinically isolated *E. coli* O177 strains using primer pairs wl-4393/wl-4394 (e), wl-4417/wl-4418 (f), 4395/wl-4396 (g), and wl-4419/wl-4420 (h). Lanes 1 and 14, DNA marker, bands with lengths of 100 bp, 250 bp, 500 bp, 750 bp, 1 kb, and 2 kb; lane 2, G1606; lane 3, G1607; lane 4, G1608; lane 5, G2042; lane 6, G2043; lane 7, G2044; lane 8, G2045; lane 9, G2046; lane 10, G2047; lane 11, G2048; lane 12, G2049; lane 13, G2068.

186 known O-antigen forms of *E. coli* and *Shigella* strains. Except for the pools containing *E. coli* O174, which gave PCR products of the expected size, no PCR products were obtained with all other pools tested (Fig. 2). The same high primer specificity was found with primers designed for *E. coli* O177 (Table 4). The primer pairs listed in Table 4 were further used in double-blind tests on *E. coli* clinical isolates including 12 *E. coli* O174 strains, 11 *E. coli* O177 strains (Table 1), and 26 *E. coli* strains of other O serogroups (Fig. 3). All of the *E. coli* O174 and O177 strains gave the expected PCR products corresponding to primer pairs used, and no PCR products were obtained from strains belonging to other O serogroups. The PCR products amplified from all O174 and O177 strains by the primers wl-4421/wl-4422 and wl-4417/wl-4418 were sequenced, and it was found that all the sequences of different strains of O174 or O177 share identity of more than 99.5% (at most, 1 base different). The results show that the primers for *E. coli* O174 and O177 are highly specific and suitable for the development of PCR assays for the identification and detection of *E. coli* O174 or O177 strains.

Sensitivity of the O-serogroup-specific PCR assays. The primer pairs described in Table 4 were used to amplify serially diluted chromosomal DNA prepared from the *E. coli* reference strains for O174 (2531-54) and O177 (E40874-85). Positive PCR results were obtained from as little as 1 pg per μl of DNA for either of the type strains.

wzx-specific primer pairs wl-4397/wl-4398/wl-4399/wl-4400 (O174) and wl-4393/wl-4394/wl-4395/wl-4396 (O177) were used for the detection of serogroup O174 and O177 reference strains from spiked samples of pork and water. By this, as few as 10^3 CFU per g was detected in samples that were examined directly, and 0.1 CFU per g was detected in samples that were further incubated at 37°C for 12 h.

The O-antigen gene-specific PCR assays developed in this study were found to be highly suitable for the detection of *E. coli* strains belonging to the novel serogroups O174 and O177. *E. coli* groups O174 and O177 were shown to comprise Shiga toxin-producing *E. coli*, EHEC, and atypical EPEC strains which were isolated from diseased humans, animals, and food in different parts of the world. At present, the O-antigen-specific PCR is the only method generally applicable for screening of *E. coli* O174 and O177 strains, since other diagnostic tools for their detection are not yet commercially available. Detection of O174 and O177 strains by PCR could be the method of choice for epidemiological investigations of outbreaks and sporadic infections and for the investigation of animals and the food chain. Last but not least, this method could be useful to investigate the association of certain O174 strains with specific animal hosts for the development of possible prevention strategies against the spread of these pathogens.

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